Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site

CAROLE L. YAUK AND JAMES S. QUINN*

Department of Biology, McMaster University, Hamilton, ON Canada L8S 4K1

ABSTRACT   Genotoxins, such as polycyclic aromatic compounds, are ubiquitous in urban and industrial environments. Our understanding of the role that these chemicals play in generating DNA sequence mutations is predominantly derived from laboratory studies with specific genotoxins or extracts of contaminants from environmental media. Most assays are not indicative of the germinal effects of exposure *in situ* to complex mixtures of common environmental mutagens. Using multilocus DNA fingerprinting, we found the mutation rate in herring gulls inhabiting a heavily industrialized urban harbor (Hamilton Harbour, Ontario) to be more than twice as high as three rural sites: Kent Island, Bay of Fundy; Chantry Island, Lake Huron; and Presqu'ile Provincial Park in Lake Ontario. Overall we found a mutation rate of 0.017 ± 0.004 per offspring band in Hamilton, 0.006 ± 0.002 at Kent Island, 0.002 ± 0.002 from Chantry Island, and 0.004 ± 0.002 from Presqu'ile Provincial Park. The mutation rate from the rural sites (pooled) was significantly lower than the rate observed in Hamilton Harbour (Fisher's exact test, two-tailed; $P = 0.0006$). These minisatellite DNA mutations may be important biomarkers for heritable genetic changes resulting from *in situ* exposure to environmental genotoxins in a free-living vertebrate species.

Levels of persistent genotoxic chemicals are elevated in the aquatic sediments and air of many areas. One possible consequence of exposure to these mutagenic compounds is an increase in germ-line mutations affecting the frequency of genetic diseases in a population. Certain chemicals have been shown to induce mutations in laboratory experiments. *Ex situ* assays are essential for establishing possible effects on species exposed to specific contaminants, but do not adequately evaluate the effects of complex mixtures encountered in nature. *In situ* techniques are typically indirect measures of mutagenicity (sister chromatid exchange, DNA adduct analyses) or measure significant biological endpoints that often result in spontaneous abortion (chromosome aberration) creating difficulty in observing significantly elevated levels in viable offspring (1). Most screenings for direct DNA sequence changes are somatic cell assays. For example, studies have shown increased mutation rates in smokers at the hypoxanthine phosphoribosyltransferase (*hprt*) locus (2) and in chemotherapy patients (3). However, even in acutely exposed individuals, mutations are extremely rare events, and a large sample size is needed to obtain statistically significant results. In addition, these somatic assays do not reveal the possible implications of exposure to future generations.

It has been difficult to establish that certain agents induce heritable mutations. In the laboratory, mouse assays, such as the mouse dominant lethal test, are used to determine the genetic effects of mutagens (4). This test evaluates mortality in the fetuses of mutagen-treated and untreated mice. A large number of animals are required to obtain significant results, and the assay does not represent biologically relevant exposure to ambient concentrations of complex mixtures of chemicals.

Induction of germ-line mutations was examined recently in humans and other animals acutely exposed to radiation. Baker *et al.* (5) estimated base pair substitution rates for the mitochondrial cytochrome *b* gene in two free-living species of voles (*Microtus arvalis* and *Microtus rossiaemeridionalis*) inhabiting an area near reactor 4 at Chernobyl, Ukraine. They found rates of substitution (0.0001 nucleotide/site/year) to be hundreds of times greater than typical mitochondrial rates [normally 10^{-6} to 10^{-8} per year; (6, 7)]. Such high rates of mutation in a protein-coding gene have never before been documented in a mammalian species. However, substitution rates in mitochondrial DNA are much higher than nuclear genes and may not reflect mutation in the nuclear genome (8). Radiation has been shown to cause DNA alterations in F_1 progeny of Japanese medaka fish (*Oryzias latipes*) at microsatellite loci (9), and at minisatellite loci in mice [*Mus musculus*; C57BL/6N, C3H/HeN, and (101/HY × C3H/SnY)F_1 hybrids; refs. 10 and 11]. Dubrova *et al.* (11) noted a statistical increase in mutation frequency in offspring of mice exposed to 0.5 Gy γ-radiation. Kodaira *et al.* (12) examined children of atomic bomb survivors at six minisatellite loci using Southern blot analysis and observed no significant difference in mutation rates in children of exposed versus unexposed parents. In a recent study following their work with mice (11), Dubrova *et al.* (13) examined the multilocus DNA fingerprints of human survivors of the Chernobyl accident who continued inhabiting a heavily polluted (radioactive and nonradioactive) area of the Mogilev district of Belaris and their children born about 9 years after the accident. They found the frequency of mutations to be twice as high in the exposed families compared with a control group from the United Kingdom. The mutation rate in the Mogilev group correlated with exposure to caesium-137 indicating radiation induction of germ-line mutations. The authors suggest that environmental mutagens resulting from agricultural or industrial processes may also play a role in the induction of germ-line mutation in addition to post-Chernobyl radioactive contamination.

Here, we report the use of multilocus DNA fingerprinting to examine *in situ* mutations in families of herring gulls (*Larus argentatus*), a sentinel species, nesting in an industrial urban harbor contaminated with nonradioactive chemicals, and from relatively uncontaminated rural sites in the Great Lakes and in the Bay of Fundy. We show a significantly higher rate of mutation in gulls exposed to potentially hazardous chemicals. Herring gulls, long-lived fish eaters distributed throughout the Northern hemisphere, are nonmigratory in the Great Lakes. Therefore, effects of contamination can be attributed to their local environment and not to exposure during migration or on more polluted wintering grounds.

MATERIALS AND METHODS

Sampling Sites. Hamilton Harbour (43°15′ N, 79°51′ W) is a natural embayment at the extreme western end of Lake Ontario. The Harbour is the site of the two largest steel mills in Canada. The water, sediment, and air in the Hamilton area are known to be contaminated with polycyclic aromatic compounds (PACs) and other chemicals showing genotoxic activity (14, 15) including heavy metals (16). Chantry Island on Lake Huron (44°29′ N, 81°23′ W) and Presqu'ile Provincial Park on Lake Ontario (44°00′ N, 77°43′ W) are two clean rural colonies within the Great Lakes. These colonies are removed from the mainland and are not situated near point sources of PACs from roads or industries. Kent Island (44°34′ N, 66°45′ W), the site of our "pristine" control colony in the Bay of Fundy, is also removed from the mainland and any point sources of PACs.

Sample Collection. Adult herring gulls were captured on their nests late in the incubation period, in 1992 and 1993. Small-volume blood samples were collected from the brachial vein of adults and from the jugular vein of chicks within 2 days of hatching. Blood was stored in 1× lysis buffer (4.0 M urea/0.2 M NaCl/0.1 M Tris·HCl, pH 8.0/0.5% n-laurylsarcosine/0.1 M 1,2-cyclohexanediamine) at 4°C. Our handling of gulls was in accordance with University animal care guidelines. We analyzed DNA from 35 nestlings and their parents from Hamilton Harbour, 47 from Kent Island, 32 from Chantry Island, and 29 from Presqu'ile Provincial Park.

Molecular Analyses. Approximately 25–50 μl of blood was digested twice with proteinase K (83.3 units at 37°C for 12 hr) and purified with two phenol/chloroform (70:30) and one chloroform extraction (17). Concentrations of DNA were determined with a TK100 minifluorometer and with agarose gel electrophoresis.

Following quantification, 15 μg of herring gull DNA was digested with HaeIII and ethanol precipitated. Three nanograms of digested λ DNA size marker (18) combined with 4.5 μg of sample DNA were size fractionated by electrophoresis for 36–48 hr at 1.25–1.5 V/cm in 28-cm long, 0.8% agarose gels. BamHI-digested adenovirus size standards were run as side lane markers. Following electrophoresis, the gels were acid nicked for 15 min (0.25 M HCl), denatured (1.5 M NaCl/0.5 M NaOH) for 1 hr and neutralized (1.5 M NaCl/0.5 M Tris base/1 M EDTA, pH 7.2) for 1 hr. DNA was transferred to a polyvinylidine membrane (Immobilon N; Millipore) by Southern blotting, and then air dried and baked to fix the DNA to the membrane (1–2 hr at 80°C).

Blots were prehybridized at 65°C with a sodium orthophosphate prehybridization solution (19). The blots were sequentially probed with four probes: Jeffreys 33.15, J33.6 (20), pSP2.5RI (PER) mouse probe homologous to the Drosophila periodic gene (21), and finally λ. Unincorporated nucleotides were separated from labeled probe with a Sepharose spin column. After overnight hybridization, blots were washed (2× standard saline citrate/0.1% SDS) at 65°C and placed on x-ray film at −70°C for both short and long exposures. After stripping, the blots were again exposed to film for a minimum of 48 hr to ensure that probe DNA was removed before subsequent probing.

Analysis. For each colony, we quantified the number of novel bands (not found in either parent's sample) between 23 and 3.5 kb in each nestling's DNA profile. The DNA fingerprints were scored without knowledge of colony location. Although herring gulls are socially monogamous, we did not make assumptions about parentage. The probability of only one novel band due to extra-pair fertilization is extremely low given the hypervariability of the loci and the number of loci examined per individual. These bands were considered the result of mutations. Average band sharing was calculated to be 0.18 based on pair-wise comparisons of 35 randomly selected

adults from two colonies (band sharing = 2 × no. of bands shared/total no. of bands scored; ref. 22). Two chicks, each with 12 nonmaternal bands, were found to have two novel bands each. The probability that a randomly selected adult (representing an extra-pair copulation) would share 10 or more of these bands was calculated to be 2.3×10^{-6} $[(\Sigma_{i=2}^{11} i \times 0.18^{10}) + 12 \times 0.18^{11} + 0.18^{12}]$, suggesting that the novel bands were mutations rather than fragments inherited from an extra-pair fertilization. Band sharing between parent and offspring for nestlings exhibiting one or two novel fragments was high (0.44–0.80; mean = 0.58 ± 0.08) compared with unrelated dyads. Five nestlings examined exhibited six or more novel bands and were not included in the analysis. Their band sharing with at least one putative parent was low (0.08–0.26), suggesting these were not their genetic parents. One nestling from Kent Island had a mutation that was detected by both J33.15 and J33.6, and one nestling from Chantry Island had a mutation that was detected by all three of the probes used. These mutations were counted as one mutational event in the determination of overall mutation rate, but were included with each probe in the calculation of mutation rate per probe. A subsample (70%) of nestlings with mutations from Hamilton Harbour were rerun to confirm that the mutant fragments were reproducible. In each case, mutations were present within the same size range. A two-tailed Fisher's exact test was used to compare the mutation rate for Hamilton Harbour to each of the cleaner sites.

RESULTS

A well-spaced banding pattern allowed the easy detection of novel bands (bands arising from length change mutations) from DNA fingerprints of herring gull families (Fig. 1). We
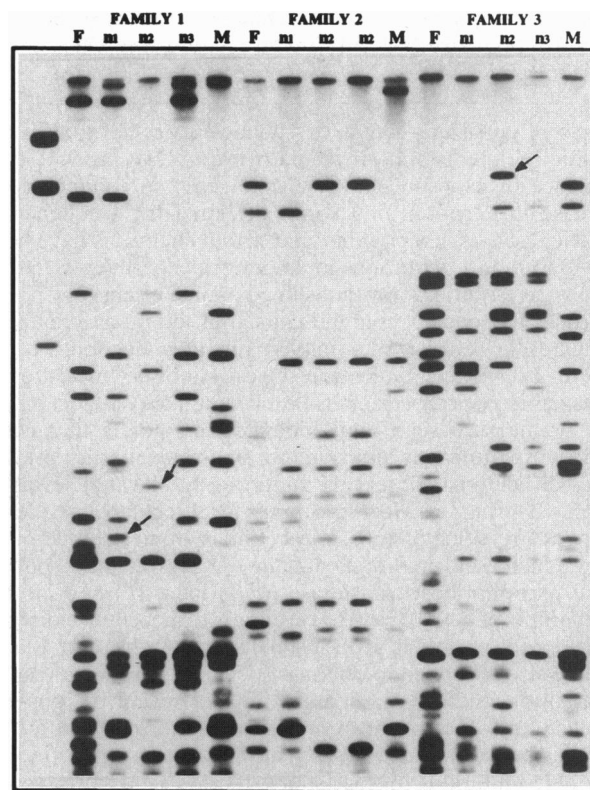

FIG. 1. DNA fingerprints of samples from three herring gull families from Hamilton Harbour digested with HaeIII and probed with J33.15. The three novel bands are indicated with arrows. F and M designate the female and male parents. The nestlings are designated n1–n3 (one nestling in family 2 was sampled twice).

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Table 1. DNA fingerprinting mutation rates in herring gull nestlings

Location	No. of mutations (no. of nestlings with mutations)	No. of nestlings scored	No. of fragments scored	Mean no. of bands scored, ±SE	Mutation rate* per fragment scored[†]
Hamilton Harbour	16 (14)	35	969	27.7 ± 0.9	0.017 ± 0.004
Kent Island	7 (7)	47	1252	26.6 ± 0.8	0.006 ± 0.002
Chantry Island	2 (2)	32	844	26.4 ± 0.9	0.002 ± 0.002
Presqu'ile Park	3 (3)	29	688	23.7 ± 1.0	0.004 ± 0.002

*Rate = (total no. novel bands/total no. bands scored) using J33.15, J33.6, and PER.
[†]SE = $[p(1 - p)/n]^{0.5}$.

identified 16 novel bands in samples from 14 individuals of the 35 nestlings examined from Hamilton Harbour, 3 novel bands from 3 nestlings of the 29 individuals sampled at Presqu'ile Provincial Park, 2 mutations in 32 nestlings from Chantry Island, and 7 novel fragments from 7 individuals of the 47 examined from Kent Island (Table 1). The mutation rate (mutations per offspring band) in Hamilton was significantly higher than the mutation rate (pooled) for the control sites (Fisher's exact test, two-tailed; $P = 0.0006$). The mutation rate in Hamilton was significantly higher than the rates calculated individually for Kent Island, Chantry Island, and Presqu'ile Provincial Park respectively (Fisher's exact test, two-tailed; $P = 0.018, P = 0.003$, and $P = 0.032$, respectively). Differences among clean colonies were not statistically significant (Fisher's exact test, two-tailed; $P = 0.328–0.994$). The mutation rates per fragment scored at the clean sites are similar to the rates found in other species, including humans (Table 2).

DISCUSSION

Herring gull nestlings from Hamilton Harbour, an extensively industrialized urban site, exhibited a significantly higher rate of mutation than three nonindustrial rural locations. The increased mutation rate may be explained by elevated concentrations of genotoxins in the aquatic sediments and air particulate at the Hamilton site. The Harbour is known to be contaminated with chemicals showing high levels of genotoxic activity. PACs, predominant genotoxins in urban and industrial areas, are abundant in the air in Hamilton and in the sediments of Hamilton Harbour (14, 15), and concentrations of heavy metals are elevated in the Harbour sediments (16). Hamilton Harbour herring gulls are exposed to contaminants from two possible sources: (*i*) airborne contaminants from coking process emissions from the steel industries and/or from vehicle emissions from a nearby heavily used highway complex, and (*ii*) chemical contaminants in their aquatic diet. Conversely, the rural Great Lakes sites, Presqu'ile Provincial Park and Chantry Island, are removed from point sources of contaminants such as heavy industries and highway complexes. Kent Island, our "pristine" colony in the Bay of Fundy, is distant from the mainland and any point sources of contaminants. Concentrations of polychlorinated biphenyls and other organochlorines in the eggs and tissue of gulls from Kent Island have been approximately one-fifth the concentration here than all other herring gull colonies analyzed in the Great Lakes for the past 15 years (G. A. Fox, personal communication). For this reason, Kent Island has been used routinely by the Canadian Wildlife Service as a control site for toxicological studies. We suggest that the current ambient levels of chemical contaminants present in the environment of the Hamilton Harbour herring gulls may be inducing significantly elevated rates of minisatellite DNA mutations. The induced mutation rate, possibly attributable to chemical contamination, was determined conservatively by comparison with the most elevated control rate (Kent Island) to be 0.011.

Currently we are investigating two competing hypotheses to explain differences in the mutation frequencies observed.

First, mutation rates in Hamilton Harbour may be elevated if the average age of adults sampled is greater than at other colonies, since older individuals may be more susceptible to mutation. Second, differences may be attributed to a highly mutable subset of alleles limited to the Hamilton Harbour population. There is a possibility that age or allelic differences may exist between Hamilton Harbour and Kent Island, given the great distance between them. It is unlikely that such differences will be true for colonies within the Great Lakes since much more mixing should occur in this small area, and climatic conditions are very similar. We are using band sharing analyses of DNA fingerprints from randomly selected adult herring gulls from each of the colonies to determine whether birds from different colonies within the Great Lakes are from the same genetic stock. In addition, we are investigating age-related mutation rates in a group of known-aged herring gulls from Presqu'ile Provincial Park. We are currently sampling gulls from other industrialized urban sites to generalize the effects of high concentrations of ambient contaminants on minisatellite DNA mutations.

It has been shown in laboratory experiments that chemical contamination affects the rate at which minisatellite mutations arise *in vitro*. Kitazawa *et al*. (26) have shown that 2-hydroxy-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) can induce fragment-length change mutations in two mouse tumor cell lines using multilocus DNA fingerprinting. They found that length change mutations arose at frequencies between 21–53% and 22–35% (control = 0%), depending on the concentration of PhIP, for the two lines, respectively, and concluded that PhIP induces recombinational mutations. Ogheri *et al*. (27) examined the induction of minisatellite DNA mutation in V79 cells treated with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG). They compared MNNG-induced mutation frequencies at the *hprt* locus to minisatellite DNA mutation frequencies examined with the J33.15 probe. They concluded that DNA mutations occurred at minisatellites much more frequently than in transcribed sequences (4–31% minisatellite mutant frequency for different concentrations of MNNG-treated cells versus 1.6–7.5% *hprt* mutant frequency). A statistically significant difference in the frequency of minisatellite mutations between MNNG-treated cells and their controls was seen. Ledwith *et al*. (28) found a 2- to 5-fold higher frequency of minisatellite DNA rearrangements in chemical carcinogen-induced mouse liver tumors compared with spontaneous tumors. They suggest that DNA fingerprinting may be used to differentiate between chemically-induced versus spontaneous tumors.

Dubrova *et al*. (13) suggest that nonradioactive contaminants present in the environment of the Chernobyl survivors may also play a role in the induction of minisatellite mutations. Their control families had mutation rates similar to those found in the Hamilton Harbour herring gull families and higher than previously reported human mutation rates with the same probes (Table 2). Ubiquitous environmental mutagens may elevate background mutation frequencies in humans inhabiting urban areas as in the United Kingdom control samples.

Table 2.   A comparison of minisatellite mutation rates per fragment scored for various species

Species	Mutation rate* (probe)	Ref. or source
L. argentatus, Hamilton Harbour	0.019 ± 0.007 (J33.15) 0.019 ± 0.007 (J33.6) 0.009 ± 0.006 (PER)	This study
L. argentatus, Kent Island	0.006 ± 0.004 (J33.15) 0.007 ± 0.004 (J33.6) 0.006 ± 0.004 (PER)	This study
L. argentatus, Chantry Island	0.006 ± 0.004 (J33.15) 0.003 ± 0.003 (J33.6) 0.004 ± 0.004 (PER)	This study
L. argentatus, Presqu'ile Park	0.012 ± 0.007 (J33.15) 0 (J33.6) 0 (PER)	This study
House sparrow *Passer domesticus*	0.004 (J33.15, J33.6)	23
Great tit *Parus major*	0.008 (J33.15, M13)	24
Blue tit *Parus caeruleus*	0.003 (J33.15, M13)	24
Humans *Homo sapiens*	0.011 (J33.15) 0.05 (J33.6)	25
Control families	0.0154 (J33.15, MS32, CEB1)	13
Chernobyl survivors	0.0303 (J33.15, MS32, CEB1)	13

*SE = $[p(1 - p)/n]^{0.5}$.

Minisatellite DNA examined with DNA fingerprinting has several advantages for the detection of genotoxin-induced mutagenesis. These tandemly repeated arrays of nucleotides show high rates of mutation compared with unique sequence DNA (29, 30), probably due to unequal sister chromatid exchange, gene conversions, or replication slippage (31, 32). Such extremely variable minisatellite DNA loci may be hypersensitive to induced instability, thereby reducing the sample sizes needed for mutation analyses (33). Furthermore, the mutations seen with DNA fingerprinting are predominantly heritable (34). Several studies have shown somatic stability of minisatellite DNA (18) and inheritance of mutant fragments in subsequent generations in humans (29). Therefore, mutations detected by minisatellite DNA analyses likely occur in the germ line or possibly very early in embryogenesis, before differentiation of somatic and germ-line tissues. Additionally, by using multilocus DNA fingerprinting we can survey several loci simultaneously, thereby increasing the statistical power to distinguish mutation rates among different sites. Finally, analyses of minisatellite DNA should be less affected by selection as minisatellites are generally in noncoding regions of the genome.

Understanding the mutagenic impact of ambient concentrations of pollutants on germ cells is of immediate concern as heritable changes, as well as somatic changes, may result in increased genetic disease. The factors responsible for elevated levels in Hamilton Harbour may vary with environmental conditions (e.g., air pollution levels vary with weather), and causal relationships remain to be determined. The higher mutation frequency in the Hamilton Harbour herring gulls is consistent with the degree of industrial contamination present at this location. Because the mutations were measured in a higher vertebrate, possessing complex enzymatic systems for the metabolism of xenobiotics, all members of this ecosystem, including humans, are potentially at risk. These heritable sequence changes appear to be relevant biomarkers that can be monitored in free-living species *in situ*.

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